OLIGONUCLEOTIDES FOR ENHANCED BIOAVAILABILITY

FIELD OF THE INVENTION

The present invention relates to oligonucleotides comprising chemical modifications which surprisingly enhance the alimentary bioavailability of oligonucleotides, and methods of using such chemically modified oligonucleotides. More particularly, the modified nucleotides of the invention are used in methods of administering oligonucleotides to the alimentary canal of an animal for investigative or therapeutic purposes. More specifically, the present invention is directed to the use of oligonucleotides administered via the alimentary canal to modulate the expression of one or more genes in an animal including a human. More specific objectives and advantages of the invention will hereinafter be made clear or become apparent to those skilled in the art during the course of explanation of preferred embodiments of the invention.

BACKGROUND OF THE INVENTION

Advances in the field of biotechnology have given rise to significant advances in the treatment of previously-intractable diseases such as cancer, genetic diseases, arthritis and AIDS. Many such advances involve the administration of nucleic acids, including oligonucleotides, to a subject, particularly a human subject. To date, however, although the administration of oligonucleotides and other nucleic acids via parenteral routes has been shown to be effective for the treatment of diseases and/or disorders (see, *e.g.*, U.S. Patent No. 5,595,978, which issued January 21, 1997 to Draper *et al.* and which discloses intravitreal injection as a means for the direct delivery of antisense oligonucleotides to the vitreous humor of the mammalian eye; and Robertson, *Nature*

Biotechnology, 1997, *15:*209 and Anon, *Genetic Engineering News*, 1997, *15:*1, both of which discuss the treatment of Crohn's disease via intravenous infusions of antisense oligonucleotides), nucleic acids have generally not been successfully administered via non-traumatic (non-parenteral) routes such as mucosal routes including oral delivery. Non-parenteral administration of oligonucleotides and other nucleic acids offers the promise of simpler, easier and less injurious administration of such nucleic acids without the need for sterile procedures. Thus, there remains a need to provide compositions and methods to enhance the alimentary availability of novel drugs such as oligonucleotides. It is desirable that such new compositions and methods provide for the simple, convenient, practical and optimal alimentary delivery of oligonucleotides and other nucleic acids.

A few preliminary studies on the pharmacokinetics and metabolism of various types of oligonucleotides *in vivo* have been published. Iversen (1991) examined the *in vivo* pharmacokinetics of phosphorothioate oligonucleotides in mice, rats and rabbits (*Anti-Cancer Drug Design*, 1991, *6:*531). Agrawal *et al.* described the pharmacokinetic results of intravenous and intraperitoneal administration of phosphorothioate oligonucleotides (*Proc. Natl. Acad. Sci. U.S.A.*, 1991, *88:*7595). Iversen (1993) discuses these and other results and, based on these early data, evaluates the potential for phosphorothioate oligonucleotides for systemic treatment (Chapter 26 *In: Antisense Research and Applications*, Crooke *et al.*, ed., CRC Press, Inc., Boca Raton, FL, 1993, pages 461-469). Crooke discusses some of the early data regarding the potential toxicology of some modified oligonucleotides (Chapter 27 *In: Antisense Research and Applications*, Crooke *et al.*, ed., CRC Press, Inc., Boca Raton, FL, 1993, pages 471-492).

International Publication No. WO 96/20732, by Song *et al.*, published July 11, 1996, discloses gene delivery vehicles ("GDVs") stated to be useful for the non-traumatic administration of nucleic acids.

International Publication No. WO 97/05267, by King, published February 13, 1997, discloses a composition for the oral delivery of polynucleotides to, *inter alia*, mucosal cells via a mucosal binding protein, GM-1.

U.S. Patent No. 5,534,496, which issued July 9, 1996, to Lee *et al.*, discloses peptide compositions stated to be useful for enhancing epithelial drug transport of a variety of agents.

U.S. Patent No. 5,591,721, which issued January 7, 1997, to Agrawal *et al.*, discloses a method of introducing an intact oligonucleotide into a mammal via oral administration, wherein the oligonucleotide comprises phosphorothioate internucleoside linkages between every nucleoside and further comprises at least two 2'-O-methyl-ribonucleotides at each end thereof.

SUMMARY OF THE INVENTION

The present invention provides oligonucleotides which have enhanced bioavailability when administered into the alimentary canal compared to a phosphorothioate oligonucleotide of substantially the same sequence. Said oligonucleotides preferably comprise at least one heteroatom backbone modification or at least one 2' sugar modification, more preferably 2'-O-alkyl or 2'-alkoxyalkoxy modifications. Methods of enhancing bioavailability of an oligonucleotide which is administered into the alimentary canal by replacing at least one backbone linkage of the oligonucleotide with a heteroatomic backbone linkage or by replacing at least one sugar moiety of the oligonucleotide with a sugar moiety which contains a 2'-O-alkyl or 2'-alkoxyalkoxy modification.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a table showing ISIS 7818, 7817 and 13251.

Figure 2 is a table showing the effect of 2'-modifications of oligonucleotides on bioavailability in mice.

Figure 3 is a table showing ISIS 4189, 14182 and 14183.

Figure 4 is a table showing bioavailability of MMI modified oligonucleotides in mice.

Figure 5 is a line drawing showing the structure of the SATE modification.

Figure 6 is a table showing the effect of the SATE modification on oligonucleotide bioavailability in mice.

Figure 7 is a table showing ISIS 5132, 13650 and 12854.

Figure 8 is a table showing rates of absorption of oligonucleotides in different sites of the rat intestine.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides compositions and methods for the alimentary delivery of nucleic acids in an animal including a human. In particular, the present invention provides compositions and methods for modulating the *in vivo* expression of a gene in an animal which involves the administration of an antisense oligonucleotide to the alimentary canal of an animal, thereby bypassing the complications and expense which may be associated with intravenous and other routes of administration. Bioavailability of oligonucleotides via alimentary administration is achieved through the use of the compositions and methods of the invention. The term "bioavailability" refers to a measurement of what portion of an administrated drug reaches the circulatory system when a non-parenteral mode of administration is used to introduce the drug into an animal. The term is used for drugs whose efficacy is related to the blood concentration achieved, even if the drug's ultimate site of action is intracellular (van Berge-Henegouwen *et al.*, *Gastroenterol.*, 1977, *73:*300). Traditionally, bioavailability studies determine the degree of intestinal absorption of a drug by measuring the change in peripheral blood levels of the drug after an oral dose (DiSanto, Chapter 76 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 1451-1458).

The oligonucleotides of the invention are formulated into a pharmaceutically acceptable composition according to methods well known in the art. A "pharmaceutically acceptable composition" or, more simply, "pharmaceutical composition," is one which, together with such excipients, diluents, stabilizers, preservatives and other ingredients as are appropriate to the nature, composition and mode of administration of the formulation, will provide the nucleic acid(s) in a proper physical form without interfering with the activity of the nucleic acid(s) and which can be introduced into the body of an animal without unacceptable side-effects or toxicity. As is known in the medical arts, a compound that is not pharmaceutically acceptable for a given patient having a particular disease or disorder may in fact be pharmaceutically acceptable to another patient with a different set of attendant circumstances. For example, a high degree of toxicity might not be acceptable for a patient suffering from a mild, non-life-threatening disorder but be nonetheless pharmaceutically acceptable for a terminally ill patient. As another example, due to differences in human physiology during development, a composition that is

pharmaceutically acceptable for most adults might be inappropriate for a child or pregnant woman.

The present invention provides pharmaceutical compositions comprising oligonucleotides which have been shown to have therapeutic utility and which contain modifications to enhance their bioavailability. The remainder of the Detailed Description relates in more detail (1) modes of administration, (2) carrier compounds, (3) oligonucleotides and other nucleic acids, and (4) formulations of the invention.

The present invention provides compositions and methods for delivery of one or more nucleic acids to the alimentary canal of an animal. For purposes of the invention, the term "animal" is meant to encompass humans as well as other mammals, as well as reptiles, amphibians, and birds. The terms "alimentary administration" or "alimentary delivery" refer to the provision of a pharmaceutical composition via the mouth through ingestion, or via some other part of the alimentary canal via a variety of routes. Thus, these terms include several routes of administration including, but not limited to, oral, rectal, endoscopic and sublingual/buccal administration. The common requirement for these modes of administration is absorption over some portion or all of the alimentary canal of the oligonucleotide(s) or other nucleic acid(s) so administered.

Delivery of a drug via the oral mucosa has several desirable features, including, in many instances, a more rapid rise in plasma concentration of the drug than via oral delivery (Harvey, Chapter 35 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, page 711). Furthermore, because venous drainage from the mouth is to the superior vena cava, this route also bypasses rapid first-pass metabolism by the liver. Both of these features contribute to the sublingual route being the mode of choice for nitroglycerin (Benet *et al.*, Chapter 1 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996, page 7).

Endoscopy can be used for drug delivery directly to a portion of the alimentary canal. For example, endoscopic retrograde cystopancreatography (ERCP) takes advantage of extended gastroscopy and permits selective access to the biliary tract and the pancreatic duct (Hirahata *et al.*, *Gan To Kagaku Ryoho*, 1992, *19(10 Suppl.)*:1591). However, the procedure is unpleasant for the patient, and requires a highly skilled staff.

Drugs administered by the oral route can often be alternatively administered by the lower enteral route, *i.e.*, through the anal portal into the rectum or lower intestine. Rectal suppositories, retention enemas or rectal catheters can be used for this purpose and may be preferred when patient compliance might otherwise be difficult to achieve (*e.g.*, in pediatric and geriatric applications, or when the patient is vomiting or unconscious). Rectal administration may result in more prompt and higher blood levels than the oral route, but the converse may be true as well (Harvey, Chapter 35 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, page 711).

The preferred method of administration is oral delivery, which is typically the most convenient route for access to the systemic circulation through the alimentary canal. Absorption from the alimentary canal is governed by factors that are generally applicable, e.g., surface area for absorption, blood flow to the ste of absorption, the physical state of the drug and its concentration at the site of absorption (Benet *et al.*, Chapter 1 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996, pages 5-7).

The co-administration of a nucleic acid with an excess of a carrier substance can result in a substantial reduction of the amount of nucleic acid recovered in the liver or kidney, presumably due to competition between the carrier substance and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioated oligonucleotide in hepatic tissue is reduced when it is co-administered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, 1995, *5:*115; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, 1996, *6:*177). The hepatic uptake system is saturated with one or more additional or alternative inactive "carrier" nucleic acids prior to administration of the active nucleic acid.

The present invention employs oligonucleotides for use in antisense modulation of the function of a nucleic acid (typically DNA or mRNA) encoding a protein the modulation of which is desired, and ultimately to regulate the amount of such a protein. Hybridization of an antisense oligonucleotide with its nucleic acid target interferes with the normal role of the nucleic acid and causes a modulation of its function in cells. For example, the functions of mRNA to be interfered with include all vital functions such as

translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with mRNA function is modulation of the expression of a protein, wherein "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of the protein. In the context of the present invention, inhibition is the preferred form of modulation of gene expression.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

An oligonucleotide is a polymer of a repeating unit generically known as a nucleotide. An unmodified (naturally occurring) nucleotide has three components: (1) a nitrogenous *base* linked by one of its nitrogen atoms to (2) a 5-carbon cyclic *sugar* and (3) a *phosphate*, esterified to carbon 5 of the sugar. When incorporated into an oligonucleotide chain, the phosphate of a first nucleotide is also esterified to carbon 3 of the sugar of a second, adjacent nucleotide. The "backbone" of an unmodified oligonucleotide consists of (2) and (3), that is, sugars linked together by phosphodiester linkages between the carbon 5 (5') position of the sugar of a first nucleotide and the carbon 3 (3') position of a second, adjacent nucleotide. A "nucleoside" is the combination of (1) a nucleobase and (2) a sugar in the absence of (3) a phosphate moiety (Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pages 4-7). The backbone of an oligonucleotide positions a series of bases in a specific order; the written representation of this series of bases, which is conventionally written in 5' to 3' order, is known as a nucleotide sequence.

Oligonucleotides may comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a particular nucleic acid. Such oligonucleotides which specifically hybridize to a portion of the sense strand of a gene are commonly described as "antisense." In the context of the invention, "hybridization" means

hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotides. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that an oligonucleotide need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a decrease or loss of function, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

Antisense oligonucleotides are commonly used as research reagents, diagnostic aids, and therapeutic agents. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes, for example to distinguish between the functions of various members of a biological pathway. This specific inhibitory effect has, therefore, been harnessed by those skilled in the art for research uses. The specificity and sensitivity of oligonucleotides is also harnessed by those of skill in the art for therapeutic uses. For example, the following U.S. patents demonstrate palliative, therapeutic and other methods utilizing antisense oligonucleotides. U. S. Patent No. 5,135,917 provides antisense oligonucleotides that inhibit human interleukin-1 receptor

expression. U.S. Patent No. 5,098,890 is directed to antisense oligonucleotides complementary to the *c-myb* oncogene and antisense oligonucleotide therapies for certain cancerous conditions. U.S. Patent No. 5,087,617 provides methods for treating cancer patients with antisense oligonucleotides. U.S. Patent No. 5,166,195 provides oligonucleotide inhibitors of Human Immunodeficiency Virus (HIV). U.S. Patent No. 5,004,810 provides oligomers capable of hybridizing to herpes simplex virus Vmw65 mRNA and inhibiting replication. U.S. Patent No. 5,194,428 provides antisense oligonucleotides having antiviral activity against influenza virus. U.S. Patent No. 4,806,463 provides antisense oligonucleotides and methods using them to inhibit HTLV-III replication. U.S. Patent No. 5,286,717 provides oligonucleotides having a complementary base sequence to a portion of an oncogene. U.S. Patent No. 5,276,019 and U.S. Patent No. 5,264,423 are directed to phosphorothioate oligonucleotide analogs used to prevent replication of foreign nucleic acids in cells. U.S. Patent No. 4,689,320 is directed to antisense oligonucleotides as antiviral agents specific to cytomegalovirus (CMV). U.S. Patent No. 5,098,890 provides oligonucleotides complementary to at least a portion of the mRNA transcript of the human *c-myb* gene. U.S. Patent No. 5,242,906 provides antisense oligonucleotides useful in the treatment of latent Epstein-Barr virus (EBV) infections. Other examples of antisense oligonucleotides are provided herein.

The oligonucleotides in accordance with this invention preferably comprise from about 8 to about 30 nucleotides. It is more preferred that such oligonucleotides comprise from about 15 to 25 nucleotides. As is known in the art, a nucleotide is a base-sugar combination suitably bound to an adjacent nucleotide through a phosphodiester, phosphorothioate or other covalent linkage. In the context of this invention, the term "oligonucleotide" includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides may be preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

Specific examples of some preferred modified oligonucleotides envisioned for this invention include those containing phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain

heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioates and those with CH_2-NH-O-CH_2, $CH_2N(CH_3)O$-CH_2 [known as a methylene(methylimino) or MMI backbone], CH_2-O-$N(CH_3)$-CH_2, CH_2-$N(CH_3)$-$N(CH_3)$-CH_2 and O-$N(CH_3)$-CH_2-CH_2 backbones, wherein the native phosphodiester backbone is represented as O-P-O-CH_2). Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Patent No. 5,034,506). Further preferred are oligonucleotides with NR-C(*)-CH_2-CH_2, CH_2-NR-C(*)-CH_2, CH_2-CH_2-NR-C(*), C(*)-NR-CH_2-CH_2 and CH_2-C(*)-NR-CH_2 backbones, wherein "*" represents O or S (known as amide backbones; DeMesmaeker *et al.*, WO 92/20823, published November 26, 1992). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen *et al.*, *Science*, 1991, *254:*1497; U.S. Patent No. 5,539,082).

The oligonucleotides of the invention may additionally or alternatively include nucleobase modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, *e.g.*, hypoxanthine, 6-methyladenine, 5-methylcytosine, 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentiobiosyl HMC, as well synthetic nucleobases, *e.g.*, 2-aminoadenine, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N^6(6-aminohexyl)adenine and 2,6-diaminopurine (Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pages 75-77; Gebeyehu, G., *et al.*, *Nucleic Acids Res.*, 1987, *15*, 4513).

The oligonucleotides of the invention may additionally or alternatively comprise substitutions of the sugar portion of the individual nucleotides. For example, oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group. Other preferred modified oligonucleotides may contain one or more substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH_3, F, OCN, OCH_3OCH_3, $OCH_3O(CH_2)_nCH_3$, $O(CH_2)_nNH_2$ or $O(CH_2)_nCH_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3; OCF_3; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; $SOCH_3$; SO_2CH_3; ONO_2; NO_2; N_3; NH_2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino;

polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. For oral administration, it has been found that oligonucleotides with at least one 2'-substituted ribonucleotide are particularly useful because of their absorption and distribution characteristics. Oligonucleotides with at least one 2'-methoxyethoxy [2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl)] (Martin *et al., Helv. Chim. Acta*, 1995, *78*:486) modification are believed to be particularly useful for oral administration. Other preferred modifications include 2'-O-alkyl modifications, such as 2'-O-methyl (also known in the art as 2'-methoxy- or 2'-O-CH_3),2'-O-propyl (known in the art as 2'-propoxy- or 2'-$OCH_2CH_2CH_3$) and substituted 2'-O-alkyl modifications such as 2'-aminopropoxy [2'-O-$(CH_2)_3NH_2$] and 2'-DMAOE [2'-O-CH_2CH_2O-$N(CH_3)_2$]. 2'-F) modifications are also preferred. Oligonucleotides with at least one 2'-substituted ribonucleotide and, at the same time, at least one 5-methylcytidine nucleotide are presently highly preferred, with 2'-methoxyethyl being presently most preferred for combination with 5-methylcytidine.

Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. The 5' and 3' termini of an oligonucleotide may also be modified to serve as points of chemical conjugation of, *e.g.*, lipophilic moieties (see immediately subsequent paragraph), intercalating agents (Kuyavin *et al.*, WO 96/32496, published October 17, 1996; Nguyen *et al.*, U.S. Patent No. 4,835,263, issued May 30, 1989) or hydroxyalkyl groups (Helene *et al.*, WO 96/34008, published October 31, 1996).

Other positions within an oligonucleotide of the invention can be used to chemically link thereto one or more effector groups to form an oligonucleotide conjugate. An "effector group" is a chemical moiety that is capable of carrying out a particular chemical or biological function. Examples of such effector groups include, but are not limited to, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A variety of chemical linkers may be used to conjugate an effector group to an oligonucleotide of the invention. As an example, U.S. Patent No. 5,578,718 to Cook

et al. discloses methods of attaching an alkylthio linker, which may be further derivatized to include additional groups, to ribofuranosyl positions, nucleosidic base positions, or on internucleoside linkages. Additional methods of conjugating oligonucleotides to various effector groups are known in the art; see, *e.g.*, *Protocols for Oligonucleotide Conjugates (Methods in Molecular Biology, Volume 26)* Agrawal, S., ed., Humana Press, Totowa, NJ, 1994.

Another preferred additional or alternative modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more lipophilic moieties which enhance the cellular uptake of the oligonucleotide. Such lipophilic moieties may be linked to an oligonucleotide at several different positions on the oligonucleotide. Some preferred positions include the 3' position of the sugar of the 3' terminal nucleotide, the 5' position of the sugar of the 5' terminal nucleotide, and the 2' position of the sugar of any nucleotide. The N^6 position of a purine nucleobase may also be utilized to link a lipophilic moiety to an oligonucleotide of the invention (Gebeyehu, G., *et al., Nucleic Acids Res.*, 1987, *15:*4513). Such lipophilic moieties include but are not limited to a cholesteryl moiety (Letsinger *et al., Proc. Natl. Acad. Sci. U.S.A.*, 1989, *86:*6553), cholic acid (Manoharan *et al., Bioorg. Med. Chem. Let.*, 1994, *4:*1053), a thioether, *e.g.*, hexyl-*S*-tritylthiol (Manoharan *et al., Ann. N.Y. Acad. Sci.*, 1992, *660:*306; Manoharan *et al., Bioorg. Med. Chem. Let.*, 1993, *3:*2765), a thiocholesterol (Oberhauser *et al., Nucl. Acids Res.*, 1992, *20:*533), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al., EMBO J.*, 1991, *10:*111; Kabanov *et al., FEBS Lett.*, 1990, *259:*327; Svinarchuk *et al., Biochimie*, 1993, *75:*49), a phospholipid, *e.g.*, di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan *et al., Tetrahedron Lett.*, 1995, *36:*3651; Shea *et al., Nucl. Acids Res.*, 1990, *18:*3777), a polyamine or a polyethylene glycol chain (Manoharan *et al., Nucleosides & Nucleotides*, 1995, *14:*969), or adamantane acetic acid (Manoharan *et al., Tetrahedron Lett.*, 1995, *36:*3651), a palmityl moiety (Mishra *et al., Biochim. Biophys. Acta*, 1995, *1264:*229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al., J. Pharmacol. Exp. Ther.*, 1996, *277:*923). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides, are disclosed in U.S. Patents Nos. 5,138,045, 5,218,105 and 5,459,255, the contents of which are hereby incorporated by reference.

The present invention also includes oligonucleotides that are substantially chirally pure with regard to particular positions within the oligonucleotides. Examples of substantially chirally pure oligonucleotides include, but are not limited to, those having phosphorothioate linkages that are at least 75% Sp or Rp (Cook *et al.*, U.S. Patent No. 5,587,361) and those having substantially chirally pure (Sp or Rp) alkylphosphonate, phosphoramidate or phosphotriester linkages (Cook, U.S. Patents Nos. 5,212,295 and 5,521,302).

It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at within a single nucleoside within an oligonucleotide. Oligonucleotide with alternating or "staggered" backbone modifications are within the scope of the invention, with alternating MMI/phosphodiester or MMI/phosphorothioate backbones being presently preferred. The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. By way of example, such "chimeras" may be "gapmers," *i.e.*, oligonucleotides in which a central portion (the "gap") of the oligonucleotide serves as a substrate for, e.g., RNase H, and the 5' and 3' portions (the "wings") are modified in such a fashion so as to have greater affinity for the target RNA molecule but are unable to support nuclease activity (*e.g.*, 2'-fluoro- or 2'-methoxyethoxy- substituted). Other chimeras include "wingmers," or "hemichimeras," that is, oligonucleotides in which the 5' portion of the oligonucleotide serves as a substrate for,

e.g., RNase H, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but is unable to support nuclease activity (*e.g.*, 2'-fluoro- or 2'-methoxyethoxy- substituted), or *vice-versa*.

The oligonucleotide compounds of the invention encompass any bioequivalent, pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the oligonucleotide compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993 or in WO 94/26764 to Imbach *et al.*

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, *66:*1). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but

otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids; for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, *etc.*; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic

acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives. Teachings regarding the synthesis of particular modified oligonucleotides are each hereby incorporated by reference from the following U.S. patents or pending patent applications, each of which is commonly assigned with this application: U.S. Patents Nos. 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; U.S. Patent No. 5,212,295, drawn to monomers for the preparation of oligonucleotides having chiral phosphorus linkages; U.S. Patents Nos. 5,378,825 and 5,541,307, drawn to oligonucleotides having modified backbones; U.S. Patent No. 5,386,023, drawn to backbone modified oligonucleotides and the preparation thereof through reductive coupling; U.S. Patent No. 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and methods of synthesis thereof; U.S. Patent No. 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; U.S. Patent No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; U.S. Patent No. 5,539,082, drawn to peptide nucleic acids; U.S. Patent No. 5,554,746, drawn to oligonucleotides having b-lactam backbones; U.S. Patent No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Patent No. 5,578,718, drawn to nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Patents Nos. 5,587,361 and 5,599,797, drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Patent No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Patent No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Patent No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; and U.S. patent application

Serial No. 08/383,666, filed February 3, 1995, and U.S. Patent No. 5,459,255, drawn to methods of synthesizing 2'-fluoro-oligonucleotides.

The formulation of compositions and their subsequent administration is believed to be within the skill of those in the art. The invention is drawn to administration of oligonucleotides having biological activity in an animal to the alimentary canal of an animal. By "having biological activity," it is meant that the oligonucleotide functions to modulate the expression of one or more genes in an animal. In the context of this invention, "to modulate" means to either effect an increase (stimulate) or a decrease (inhibit) in the expression of a gene. Such modulation can be achieved by a variety of mechanisms known in the art, including but not limited to transcriptional arrest; effects on RNA processing (capping, polyadenylation and splicing) and transportation; enhancement of cellular degradation of the target nucleic acid; and translational arrest (Crooke *et al.*, *Exp. Opin. Ther. Patents*, 1996, *6:*1).

In an animal other than a human, the compositions and methods of the invention can be used to study the function of one or more genes in the animal. For example, antisense oligonucleotides have been systemically administered to rats in order to study the role of the *N*-methyl-D-aspartate receptor in neuronal death, to mice in order to investigate the biological role of protein kinase C-a, and to rats in order to examine the role of the neuropeptide Y1 receptor in anxiety (Wahlestedt *et al.*, *Nature*, 1993, *363:*260; Dean *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1994, *91:*11762; and Wahlestedt *et al.*, *Science*, 1993, *259:*528, respectively). In instances where complex families of related proteins are being investigated, "antisense knockouts" (*i.e.*, inhibition of a gene by systemic administration of antisense oligonucleotides) may represent the most accurate means for examining a specific member of the family (see, generally, Albert *et al.*, *Trends Pharmacol. Sci.*, 1994, *15:*250).

The compositions and methods of the invention are also used therapeutically, *i.e.*, to provide therapeutic, palliative or prophylactic relief to an animal, including a human, suffering from or predisposed to develop a disease or disorder. The term "disease or disorder" (1) excludes pregnancy *per se* but not autoimmune and other diseases associated with pregnancy; (2) includes any abnormal condition of an organism or part, especially as a consequence of infection, inherent weakness, environmental stress, that impairs normal physiological functioning; and (3) includes cancers and tumors.

In general, for therapeutic applications, a patient (*i.e.*, an animal, including a human, having or predisposed to a disease or disorder) is administered one or more nucleic acids, including oligonucleotides, in accordance with the invention in a pharmaceutically acceptable carrier in doses ranging from 0.01 ug to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the nucleic acid may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the nucleic acid is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. For example, in the case of in individual known or suspected of being prone to an autoimmune or inflammatory condition, prophylactic effects may be achieved by administration of preventative doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every

20 years. In like fashion, an individual may be made less susceptible to an inflammatory condition that is expected to occur as a result of some medical treatment, *e.g.*, graft versus host disease resulting from the transplantation of cells, tissue or an organ into the individual.

The present invention also includes pharmaceutical compositions and formulations which include the oligonucleotides of the invention. The compounds of the invention may be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference in its entirety.

Compositions and formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Formulations for non-parenteral administration of nucleic acids may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic carrier substances suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium sterate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings flavorings and/or aromatic substances and the like which do not deleteriously react with the nucleic acid(s) of the formulation. Aqueous suspensions may contain substances which increase the viscosity of the suspension

including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. Optionally, the suspension may also contain stabilizers.

Pharmaceutical compositions and/or formulations comprising the oligonucleotides of the present invention may also include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, *8*:91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, *7*:1). One or more penetration enhancers from one or more of these broad categories may be included. Penetration enhancers are described in pending United States patent application 08/886,829, filed on July 1, 1997, ~~United States patent X,XXX,XXX~~, and pending United States patent application 08/961,469, filed on October 31, 1997, United States patent 6,083,923 ~~X,XXX,XXX~~, both of which are commonly owned with the instant application and both of which are herein incorporated by reference.

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arichidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, *7*:1; El-Hariri *et al.*, *J. Pharm. Pharmacol.*, **1992**, *44*:651).

Preferred penetration enhancers are disclosed in pending United States patent application 08/886,829, filed on July 1, 1997, ~~United States patent X,XXX,XXX~~, which is commonly owned with the instant application and which is herein incorporated by reference.

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any

of the naturally occurring components of bile as well as any of their synthetic derivatives. Preferred bile salts are described in pending United States patent application 08/886,829, filed on July 1, 1997, ~~United States patent X,XXX,XXX~~, which is commonly owned with the instant application and which is herein incorporated by reference. A presently preferred bile salt is chenodeoxycholic acid, sodium salt (CDCA)(Sigma Chemical Company, St. Louis, MO), generally at concentrations of 0.5 to 2%.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations. Preferred combinations include CDCA combined with sodium caprate or sodium laurate (generally 0.5 to 5%).

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, *7:*1; Buur *et al.*, *J. Control Rel.*, **1990**, *14:*43). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi *et al.*, *J. Pharm. Phamacol.*, 1988, *40:*252).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, 1987, *39:*621).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The co-administration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a

substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioated oligonucleotide in hepatic tissue is reduced when it is co-administered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, 1995, *5:*115; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, 1996, *6:*177).

In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrates (*e.g.*, starch, sodium starch glycolate, *etc.*); or wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Patents Nos. 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, *e.g.*, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the antisense compounds of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the compounds and/or to target the compounds to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see, generally, Chonn *et al.*, *Current Op. Biotech.*, 1995, *6*, 698). Some aspects of liposome preparation are described in pending United States patent application 08/961,469, filed on October 31, 1997, United States patent 6,083,923 ~~X,XXX,XXX,~~ which is commonly owned with the instant application and which is herein incorporated by reference.

In one embodiment of the invention, a nucleic acid is administered via the rectal mode. In particular, compositions for rectal administration include foams, solutions (enemas) and suppositories. Rectal suppositories for adults are usually tapered at one or both ends and typically weigh about 2 g each, with infant rectal suppositories typically weighing about one-half as much, when the usual base, cocoa butter, is used (Block, Chapter 87 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990).

In a preferred embodiment of the invention, one or more nucleic acids are administered via oral delivery. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, troches, tablets or SECs (soft elastic capsules or "caplets"). Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, carrier substances or binders may be desirably added to such formulations. The use of such formulations has the effect of delivering the nucleic acid to the G.I. tract for exposure to the gastrointestinal mucosa. Accordingly, the formulation can consist of material effective in protecting the nucleic acid from pH extremes of the stomach, or in releasing the nucleic acid over time, to optimize the delivery thereof to the gastrointestinal mucosa. Enteric coatings for acid-resistant tablets, capsules

and caplets are known in the art and typically include acetate phthalate, propylene glycol and sorbitan monoleate.

Various methods for producing formulations for oral-gastrointestinal delivery are well known in the art (see, generally, Nairn, Chapter 83; Block, Chapter 87; Rudnic *et al.*, Chapter 89; Porter, Chapter 90; and Longer *et al.*, Chapter 91 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990).

The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of nucleic acid-containing formulation which is effective to achieve an intended purpose. Although individual needs may vary, determination of optimal ranges for effective amounts of formulations is within the skill of the art. Human doses can be extrapolated from animal studies (Katocs *et al.*, Chapter 27 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a formulation, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition, weight, extent of disease or disorder of the recipient, frequency of treatment and the nature and scope of the desired effect (Nies *et al.*, Chapter 3 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996).

As used herein, the term "high risk individual" is meant to refer to an individual for whom it has been determined, via, *e.g.*, individual or family history or genetic testing, has a significantly higher than normal probability of being susceptible to the onset or recurrence of a disease or disorder. As art of treatment regimen for a high risk individual, the individual can be prophylactically treated to prevent the onset or recurrence of the disease or disorder. The term "prophylactically effective amount" is meant to refer to an amount of a formulation which produces an effect observed as the prevention of the onset or recurrence of a disease or disorder. Prophylactically effective amounts of a formulation are typically determined by the effect they have compared to the effect observed when a second formulation lacking the active agent is administered to a similarly situated individual.

Antisense compounds with various chemical modifications have been tested in various assays for gastrointestinal uptake and bioavailability. The following descriptions are illustrative and are not intended to limit the invention.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1: Effect of 2' modifications on bioavailability after gastrointestinal administration in mice

An antisense oligonucleotide (CAGCCATGGTTCCCCCCAAC, SEQ ID NO:1) targeted to mouse protein kinase C-α (PKCα) has previously shown good activity as a phosphorothioate, and is able to inhibit PKCα expression both in vitro and in mice. Gapped versions of this sequence were synthesized with 2'-O-methyl (2'-O-Me), 2'-O-propyl (2'-O-Pro) or 2'-methoxyethoxy (2'-MOE) modifications in the flanking regions. These are shown in Figure 1. All 2'-MOE cytosines were 5-methylcytosines. Figure 2 shows the enhancing effect of these modifications on bioavailability in mice. Phosphorothioates give, in general, less than 1% bioavailability. Another oligonucleotide, ISIS 11159 (SEQ ID NO: 2), which is targeted to the 5' cap of the rat intercellular adhesion molecule 1 (ICAM-1) mRNA, was also tested. This compound is a phosphorothioate with a 2'-MOE at every sugar except the 3' terminal sugar which was a deoxy (for ease of synthesis). This compound was tested in the same way and demonstrated a radiolabel bioavailability of 21.74%. This compound is therefore preferred. ISIS 3521 (GTTCTCGCTGGTGAGTTTCA SEQ ID NO: 3) is a phosphorothioate oligodeoxynucleotide targeted to human PKCα. This sequence is presently in clinical trials for treatment of a variety of solid tumors in humans and is therefore preferred. ISIS 13312 (SEQ ID NO: 4; GCGTTTGCTCTTCTTCTTGCG) is a 2'-MOE gapped version of fomivirsen (ISIS 2922), an oligonucleotide drug targeted to cytomegalovirus (CMV) which has shown clinical efficacy in Phase III clinical trials. The seven nucleotides at the 5' end of oligonucleotide ISIS 13312 are 2'-MOE. The central seven nucleotides are deoxynucleotides, and the remaining nucleotides have 2'-O-(2-methoxy)ethyl modification, except for the last nucleotide at the 3' end which is a deoxynucleotide for ease of synthesis. All cytosines in the molecule are 5-methyl-cytosines and all intersugar linkages are phosphorothioates. This compound has been shown to have good antiviral activity and is extremely stable in the body, and is therefore preferred. Oligonucleotides have been designed to target the G-to-A mutation of the mutant codon 717 of β-amyloid precursor protein (βAPP), which mutation has been implicated in Alzheimers disease. Gapped

oligonucleotides having 2'-O-alkyl modifications are believed to be particularly useful and are therefore preferred. One such preferred sequence is SEQ ID NO: 5 (AGGTGATGAAGATCACTGT).

Example 2: Effect of MMI backbone modifications on bioavailability after gastrointestinal administration in mice

SEQ ID NO: 1 was also synthesized as a gapped oligonucleotide with alternating MMI/P=O backbone linkages throughout the molecule or in the flanking regions only. These compounds are shown in Figure 3. Such modifications enhance the bioavailability of these oligonucleotides in mice, as shown in Figure 4.

Oligonucleotides targeted to human Ha-ras have also been made with MMI backbones. ISIS 2503 (SEQ ID NO: 6) is targeted to the translation initiation codon and ISIS 2570 (SEQ ID NO: 7) is targeted to the mutant codon 12 in activated ras. These compounds are also expected to show enhanced bioavailability.

Example 3: Effect of prodrug modifications on bioavailability after gastrointestinal administration in mice

A thymidine homopolymer (T_{12}) was synthesized with either a P=S or P=O backbone with a SATE moiety (Imbach et al., WO Publication 94/26764) at each internucleoside linkage. This moiety is shown in Figure 5. The enhancing effect of this modification on bioavailability is shown in Figure 6.

Example 4: Effect of 2'-MOE modifications on the rate of absorption in rat intestine

An oligonucleotide targeted to human C-raf (SEQ ID NO. 8; TCCCGCCTGTGACATGCATT) has been shown to be extremely effective in inhibiting raf expression in vitro and in vivo and the phosphorothioate version of this sequence (ISIS 5132) is presently in human clinical trials. This sequence was tested as a phosphorothioate and as two 2'-MOE gapped oligonucleotides (shown in Figure 7) to determine their rates of absorption in different sites of the rat intestine: the duodenum/jejunum, the ileum and the colon. As shown in Figure 8, the rate of absorption was greatly enhanced in the 2'-MOE oligonucleotides compared to the P=S deoxyoligonucleotide, ISIS 5132.

Example 5: Effect of 2'-methoxyethoxy m difications on plasma c ncentration following intestinal perfusion

Rats were perfused with the compounds shown in Figure 7. After 30 minutes of perfusion, the total oligonucleotide portal plasma concentration was determined. ISIS 12854 gave portal plasma concentrations of 1.3 and 1.4 ug-eq/ml in the ileum and colon, respectively, compared to ISIS 5132 which was present at 0.1 and 0.2 ug-eq/ml. ISIS 13650 was found to be present at 0.2-0.4 ug-eq/ml.

Example 6: Effect of 2'-methoxyethoxy modifications with 5-methylcytidine modifications on in vivo bioavailability

Several oligonucleotide compounds targeted to human ICAM-1 were administered to rats by intrajejunal instillation in a modification of the procedure described in Example 2. All are uniform phosphorothioates and all have SEQ ID NO: 9 (GCCCAAGCTGGCATCCGTCA).

The compounds were:

ISIS 2302 GCCCAAGCTGGCATCCGTCA all 2'-deoxy

ISIS 14725 GCCCAAGCTGGC**ATCCGTCA** **bold** are 2'-MOE.
All 2'-MOE cytidines are 5-methylcytidines.

ISIS 15839 GCCCAAGCTGGC**ATCCGTCA** **bold** are 2'-MOE
All cytidines (including deoxycytidines) are 5-methylcytidines.

Oligonucleotides were administered at 40 mg/kg in a volume of 0.5 mL, with and without penetration enhancer(s). Oligonucleotide concentrations were measured as described in Example 4. The results are as shown in Table 1.

Table 1

In vivo bioavailability (BAV)- Plasma AUC or Tissue

Compound	Formulation	Plasma BAV	Tissue BAV
2302	Water or saline	1-2%	1-2%
	Bile salt (2% CDCA)	11%	ND
	Bile salt (2% CDCA) + fatty acids (4% Na caprate = 4% Na laurate)	14.6%	18-30%
15839	Water or saline	ND	ND
	Bile salt (2% CDCA)	17.5%	ND
	Bile salt (2% CDCA) + fatty acids (4% Na caprate = 4% Na laurate)	31.6%	ND
14725	Water or saline	5-8%	5.2%

ND=not determined

Example 7: In vivo Bioavailability of ICAM-1 oligonucleotides in Dogs

ISIS 2302 and ISIS 15839 were administered intrajejunally to "ported" dogs at oligonucleotide doses of 10 mg/kg with or without penetration enhancers. Bile salts (CDCA) were used alone or in combination with fatty acids (2% CDCA, 4% Na caprate, 4% Na laurate). Blood samples were collected and evaluated for the presence and concentration of intact oligonucleotides by CGE. Percent bioavailability (%BAV) is calculated as (intact plasma concentration area under curve by alimentary administration/intact plasma concentration by intravenous administration) x 100. Results are shown in Table 2:

Table 2

Absolute bioavailability of oligonucleotides in dogs after intrajejunal administration

Compound	Formulation	%BAV
2302	Water or saline	0.3%
		1.3%
		8.4%
15839	Water or saline	1.5%
	Bile salt (2% CDCA)	ND
	Bile salt (2% CDCA) + fatty acids (4% Na caprate = 4% Na laurate)	18%

ND=not determined

These results confirm and extend the results from the rat experimental systems.

Example 8: Synthesis and characterization of oligonucleotides

Oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. ß-cyanoethyldiisopropyl-phosphoramidites were purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-O-methyl phosphorothioate oligonucleotides were synthesized using 2'-O-methyl ß-cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base is increased to 360 seconds. The 3'-base used to start the synthesis was usually a 2'-deoxyribonucleotide for ease of synthesis.

Oligonucleotides with 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$) substituents may be synthesized according to the method of Martin, *Helv. Chim. Acta* **1995**, 78,486-504. The 3'-base used to start the synthesis may be a 2'-deoxyribonucleotide for ease of synthesis. 5-methyl-2'-deoxycytidine phosphoramidites are commercially available (Glen

Research, Sterling VA). All 2'-methoxyethoxy cytosines were 5-methyl cytosines, for which monomers were synthesized according to the following procedures.

The following examples describe the synthesis of 2'-methoxyethoxy-5-methyl cytosine monomers:

1. 2,2'-Anhydro[1-(β-D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2. 2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

3. 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

4. 3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

5. 3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside.

Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

6. 2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

7. N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using

EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

8. N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:

N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

Synthesis of oligonucleotides with methylene(methylimino) (MMI) internucleoside linkages: The introduction of MMI linkages into the oligonucleotide backbone has been shown to increase affinity of the oligonucleotide for its RNA target, and also to enhance nuclease resistance. The MMI linkage has also been shown to confer nuclease resistance to adjacent P=O linkages within an oligonucleotide. Vasseur et al., *J. Am. Chem. Soc.* **1992**, 114, 4006-4007.

Nucleotide dimers containing MMI linkages were synthesized according to the method of Vasseur et al., *J. Am. Chem. Soc.* **1992**, 114:4006. Oligonucleotides containing MMI backbone-substituted dimers were synthesized and purified by HPLC.

Prodrugs equipped with SATE moieties were synthesized according to Imbach et al., WO Publication 94/26764.

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides were purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 45 mM Tris-borate buffer, pH 7.0. Oligodeoxynucleotides and phosphorothioate

oligonucleotides were judged from electrophoresis to be greater than 80% full length material.

Example 9: Bioavailability studies

Bioavailability is determined after administration by IV bolus vs. intraduodenal instillation. For implantation of intraduodenal catheters, animals are anesthetized and a small incision is made in the abdomen. A medical grade silastic catheter is inserted through the stomach wall up the pylor and further inserted in the duodenum. The catheter is secured in place with a suture and brought subcutaneously to the exteriorization point at the base of the neck. The abdomen is irrigated with warm normal saline and penicillin G sodium. The stomach insertion and abdominal sites are closed with interrupted mattress sutures and the exteriorization point with a purse stitch. A jacket is placed on the animal to secure the cannula.

For bolus IV injection, a single bolus intravenous injection via a tail vein is administered at a target level of 3 mg/kg (15 uCi/kg) and a dose volume of 3 ml/kg. Single intraduodenal doses are given at target levels of 30 mg/kg (60 uCi/kg) at a dose volume of 3 ml/kg or one to three repeat doses at 10 mg/kg/dose (20 uCi/kg/dose) and 1 ml/kg/dose.

Animals are euthanized by exsanguination following anesthetization and whole blood is collected. In addition, tissues are collected for analysis.

Example 10: Intestinal perfusion in rats

The rat *in situ* intestine circulating perfusion technique was conducted according to Geary et al. (1991) Biopharmaceutics & Drug Disposition, 12:261-274. Rat intestinal segments (jejunum, ileum and colon) can be perfused independently. Sprague-Dawley rats were anesthetized. The proximal cannula was inserted through the stomach wall past the pyloric sphincter for experiments which included bile flow. For experiments which excluded bile flow, rats were cannulated approximately 3 cm distal to the pyloric sphincter. Concomitant sampling of blood was accomplished by insertion of a catheter in the portal vein via the ileocecal vein. Blood samples were taken at intervals and samples of the circulating perfusate were also taken. The perfusate reservoir was maintained at a

constant temperature of 37.5° and stirred at all times. The drug solutions were perfused through the intestinal segment at a constant rate of 5 ml/min.

Example 11: Determination of oligonucleotide concentration

Drug concentrations in plasma were measured using capillary gel electrophoresis. Leeds et al.(1996) Anal. Biochem. 235:36-43. Drug concentrations in tissues were measured using solid phase extraction followed by capillary gel electrophoresis. Crooke et al., J. Pharmacol. Exp. Ther. 277:923-937; Leeds et al.(1996) Anal. Biochem. 235:36-43.

Example 12: Drug instillation in dogs

Dogs were "ported" with intestinal access catheters through which formulated drug formulations (solutions or suspensions) may be introduced into various areas of the gut. Target areas include the proximal jejunum and distal ilium or the iliocecal junction. In addition to ported dogs, naive dogs are used for the assessment of formulations given by conventional routes, *e.g.*, oral administration for oral dosage forms, rectal administration for enema or suppository formulations, *etc.* Dogs were dosed at 10 mg/kg of oligonucleotide and blood samples were collected and evaluated for the presence and concentration of oligonucleotides. The absolute bioavailability was calculated.